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Assisted folding of D-glyceraldehyde-3-phosphate dehydrogenase by trigger factor

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Abstract

The *Escherichia coli* trigger factor is a peptidyl–prolyl *cis–trans* isomerase that catalyzes proline-limited protein folding extremely well. Here, refolding of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of trigger factor was investigated. The regain of activity of GAPDH was markedly increased by trigger factor after either long- or short-term denaturation, and detectable aggregation of GAPDH intermediates was prevented. In both cases, time courses of refolding of GAPDH were decelerated by trigger factor. The reactivation yield of GAPDH showed a slow down-turn when molar ratios of trigger factor to GAPDH were above 5, due to tight binding between trigger factor and GAPDH intermediates. Such inactive bound GAPDH could be partially rescued from trigger factor by addition of reduced αLA as competitor, by further diluting the refolding mixture, or by disrupting hydrophobic interactions in the complexes. A model for trigger factor assisted refolding of GAPDH is proposed. We also suggest that assisted refolding of GAPDH is due mainly to the chaperone function of trigger factor.

Keywords: chaperone; prolyl isomerase; protein folding; trigger factor

The multifunctional *Escherichia coli* trigger factor was originally identified as being involved in the maintenance of a translocation-competent conformation of the precursor protein proOmpA (outer membrane protein A) in a cell free translation system (Crooke & Wickner, 1987), and stoichiometric complexes of trigger factor and proOmpA were isolated (Crooke et al., 1988). However, contradictory results were obtained when using genetically engineered strains of *E. coli* that either over- or underproduced trigger factor, when it was found that the membrane transport of proOmpA was not affected (Guthrie & Wickner, 1990). Trigger factor was subsequently identified as a peptidyl–prolyl *cis–trans* isomerase (Stoller et al., 1995; Hesterkamp et al., 1996) and was detected in the 50s subunit of functional ribosomes known to contain the peptidyl transferase center, which covers the exit domain of the nascent polypeptide chain (Hesterkamp et al., 1996). Cooperation of enzymatic and chaperone functions makes trigger factor more effective than cyclophilins (CyPs), FK506 binding proteins (FKBPs), or the parvulin family in the catalysis of prolyl limited protein folding (Scholz et al., 1997). The groups of Luirink and Bukau have successfully cross-linked presecretory and nonsecretory proteins to trigger factor while still associated with the ribosome (Valent et al., 1995; Hesterkamp et al., 1996). Further, trigger factor has been shown to be an important cofactor in GroEL-dependent protein degradation in *E. coli* and to promote binding of GroEL to unfolded proteins (Kandror et al., 1995, 1997). Trigger factor may also be a rate-limiting component in the degradation of abnormal proteins. More recently, trigger factor from *Bacillus subtilis* was reported to catalyze in vitro protein folding and to be necessary for viability under starvation conditions (Göthel et al., 1998; Lyon et al., 1998). There is ample evidence that trigger factor plays an important and multifunctional role during protein synthesis in vivo, although precisely how it works remains unclear.

GAPDH from rabbit muscle is a homo-tetramer with a monomeric molecular weight of 36 kDa and contains 11 prolines per subunit. A crystal structure is not yet available. The spontaneous reactivation of guanidine-denatured GAPDH decreases sharply with increasing protein concentration. During refolding, GAPDH is particularly prone to aggregation, especially at high concentrations, concomitant with its sharp decrease in spontaneous reactivation (Teipel & Koshland, 1971; Cai et al., 1994). It appears that aggregation of the enzyme molecule competes with correct regain of structure and leads to low yields of reactivation. To understand the relationship between peptidyl prolyl isomerase activity and chaperone activity of trigger factor during assisted protein folding, we performed two different types of refolding experiments, using GAPDH as a substrate for either trigger factor or cyclophilin. In

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Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; αLA, α-lactalbumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EG, ethylene glycol; GdnHCl, guanidine hydrochloride; CyP, cyclophilin.

the first set of experiments, GAPDH was initially unfolded overnight at 4 °C to produce molecules with prolyl peptide bonds in thermodynamic *cis/trans* equilibrium. In the second set of experiments, GAPDH was initially unfolded for only 10 s at 4 °C to produce molecules that had not yet undergone prolyl isomerization (Freskgård et al., 1992; Kern et al., 1995). The kinetics of folding and renaturation yields of GAPDH were then compared. Finally, the reactivation of GAPDH when molar ratios of trigger factor to GAPDH were above 5 was investigated in detail, and a general pathway of refolding of GAPDH in the presence of trigger factor is proposed.

Results

Effects of trigger factor on the reactivation of GAPDH after long-term denaturation

Trigger factor is stable under all conditions used. Dilution of long-term denatured GAPDH in the presence of trigger factor, where the molar ratio of trigger factor/GAPDH was lower than 5, resulted in increasing recovery of activity with increasing ratios of trigger factor/GAPDH. Of the concentrations of GAPDH studied, namely 0.2, 1, and 2.73 μM, more marked effects of trigger factor on its reactivation were observed at the higher concentrations. As the tetrameric GAPDH dissociates to monomer in 3 M GdnHCl, concentrations of GAPDH are expressed as concentration of monomer for the convenience of comparison to trigger factor. As shown in Figure 1, when the ratio of trigger factor/GAPDH was increased from 0 to 5, the reactivation yields of GAPDH increased from 43 to 76%, 19 to 78%, and 7 to 57% corresponding to GAPDH concentrations of 0.2, 1, and 2.73 μM, respectively. This shows that trigger factor can improve significantly the reactivation of denatured GAPDH.

It is interesting to note that the recovery of activity of GAPDH was not found to increase continuously or reach a saturated value with further increase in the concentration of trigger factor. Instead, the reactivation curve showed a slow down turn when molar ratios of trigger factor to GAPDH were above 5, indicating that high concentrations of trigger factor actually suppress reactivation of GAPDH.

Cyclophilin, another peptidyl–prolyl *cis–trans* isomerase, was used as a comparison to dissect out the isomerase and chaperone activities of trigger factor. Increasing concentrations of cyclophilin showed no effect or even a slight decrease in the extent of GAPDH reactivation.

The time courses of reactivation of long-term denatured GAPDH in the presence of different concentrations of trigger factor or cyclophilin are compared in Figure 2. Theoretically, if the increased reactivation yields of GAPDH in the presence of trigger factor were due to catalysis by trigger factor as an enzyme, then the reactivation process of GAPDH should be accelerated by trigger factor. However, as shown in Figure 2, the rates of reactivation of denatured-GAPDH in the presence of trigger factor are found to be slower than that of spontaneous reactivation. The rate constants of reactivation in the presence of 0, 6, and 30 μM trigger factor were $(7.68 \pm 0.72) \times 10^{-2}$, $(5.49 \pm 0.42) \times 10^{-2}$, and $(1.43 \pm 0.17) \times 10^{-2}$ min^{-1}, respectively. Fifteen micromolars of cyclophilin had no effect on the kinetic process of the reactivation of GAPDH. It is clear that, accompanying an increase in reactivation yields, the reactivation rates decrease with increasing concentration of trigger

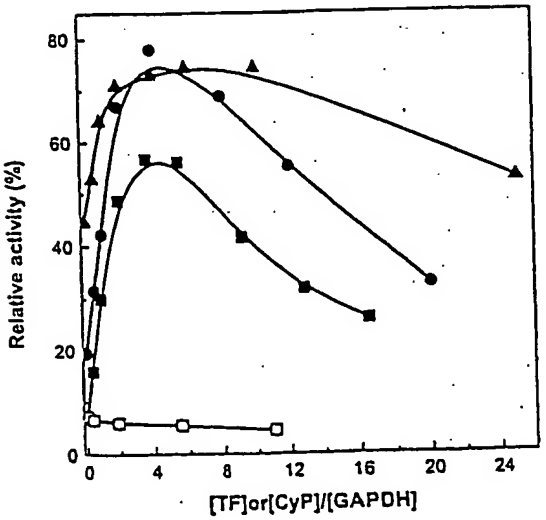


Fig. 1. Reactivation of long-term denatured GAPDH in the presence of various amounts of trigger factor. Refolding of 3 M GdnHCl-denatured GAPDH was initiated by a 50-fold dilution into 0.1 M K-Pi buffer, pH 7.5. The reactivation mixtures were first kept at 4 °C for 30 min and then for a further 3 h at 25 °C before samples were taken for assay of activity. Data are presented as the percentage of GAPDH refolded with respect to an undenatured sample of GAPDH otherwise treated in exactly the same way. (▲), (●), and (■) represent 0.2, 1, and 2.73 μM GAPDH, respectively, in the presence of trigger factor (TF). (□) represents 2.73 μM GAPDH in the presence of cyclophilin.

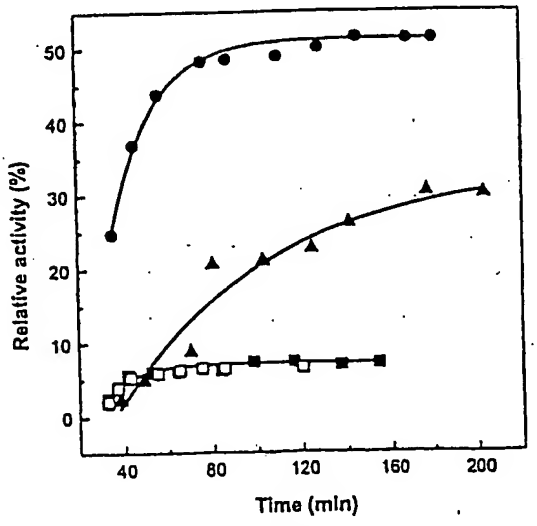


Fig. 2. Refolding kinetics of long-term denatured GAPDH in the presence of different concentrations of trigger factor at 25 °C. The refolding was followed by the regain of enzyme activity at final concentrations of GAPDH of 2.73 μM. (■), (●), and (▲) were in the presence of 0, 6, and 30 μM trigger factor, respectively. (□) represents time course of reactivation of GAPDH in the presence of 15 μM cyclophilin. Upon dilution, a 10 μL sample of the solution was withdrawn and assayed for GAPDH activity at the times indicated. There was no detectable activity during the first 30 min of incubation at 4 °C, therefore in curve fitting, transfer to 25 °C is taken as the zero time point.

factor. An increase in refolding yields and slowing down of refolding rates is a characteristic of chaperone-like activity.

Suppression of aggregation of GAPDH by increasing trigger factor concentrations

The long-term denatured enzyme in the absence of trigger factor aggregated rapidly upon dilution, as monitored by light scattering. Light scattering started to increase within 10 min of dilution and approached a constant value at ~1 h. The intensity of scattered light dropped greatly in the presence of trigger factor. With increasing concentrations of trigger factor, both the rate and the extent of aggregation were markedly inhibited (Fig. 3A,B). No light scattering change was observed at ratios of trigger factor/GAPDH above 11. In contrast, cyclophilin, at a concentration of 5.5 times that of GAPDH, increased the extent of aggregation slightly as compared with GAPDH alone. Trigger factor or cyclophilin alone showed no scattered light under the same conditions.

It is noteworthy that aggregation was suppressed when the molar ratios of trigger factor/GAPDH were >5, although the reactivation yields decreased. It is likely that folding to the native state is retarded by the formation of complexes between trigger factor and intermediates of GAPDH.

Effects of trigger factor on the reactivation of GAPDH after short-term denaturation

Unfolded molecules of GAPDH with the prolyl peptide bonds still in their native conformation were produced by a short 10 s unfolding pulse in 3 M GdnHCl at 4 °C, and renaturation was then performed in the presence of different concentrations of trigger factor and cyclophilin. After short-term denaturation, no activity of GAPDH remained and most of the secondary and tertiary structures of the native protein were lost as judged by intrinsic fluorescence and circular dichroism measurements (data not shown). The spontaneous reactivation of 10 s denatured enzyme increased to 25% (Fig. 4), compared to <10% for longer denaturation times (Fig. 1), indicating that the refolding of GAPDH was facilitated by the residual structure after incomplete denaturation. Reactivation yields in the presence of trigger factor increased with increasing ratios of trigger factor to GAPDH, reaching 72% at a trigger factor/GAPDH ratio of 4. With further increase in trigger factor/GAPDH, the reactivity declined as described for that after long-term denaturation. As was found for long-term denaturation, after short-term denaturation cyclophilin has no effect on the renaturation yield of GAPDH.

Time courses of reactivation of short-term denatured GAPDH in the presence of different concentrations of trigger factor or cyclophilin are compared in Figure 5. The rate constant of spontaneous

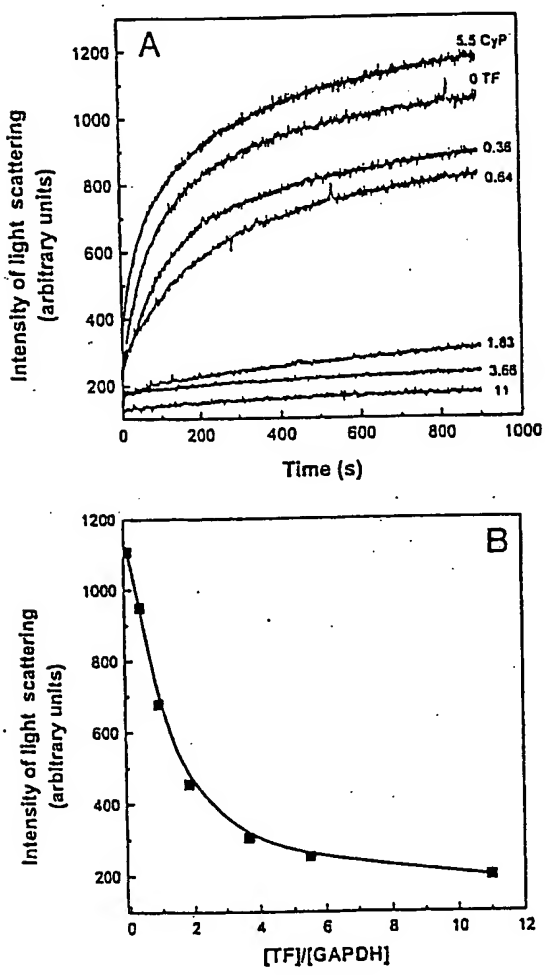


Fig. 3. Suppression of aggregation of GAPDH by increasing trigger factor concentrations. Aggregation of GAPDH upon dilution at 20 °C was monitored continuously by 90° light scattering at 488 nm. A: The time course of light-scattering change with different ratios of trigger factor to GAPDH as indicated. The concentration of GAPDH was 2.73 μM. B: Effect of the trigger factor to GAPDH ratio on the eventual levels of aggregation, determined 2 h after dilution.

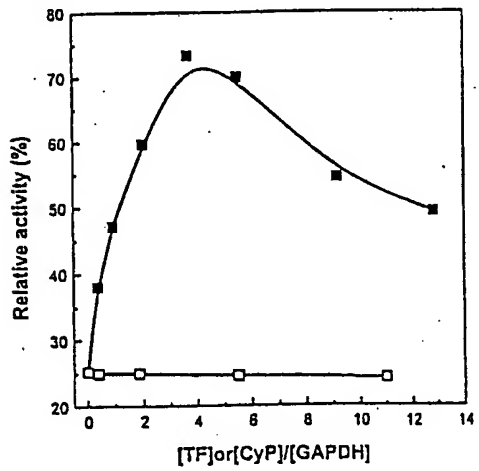


Fig. 4. Reactivation of short-term denatured GAPDH in the presence of trigger factor (■) or cyclophilin (□). The time of denaturation was limited to only 10 s in 3 M GdnHCl, after which reactivation of GAPDH was initiated by 50-fold dilution in the presence of different concentrations of trigger factor or cyclophilin. All other operations were the same as for Figure 1. The final concentration of GAPDH was 2.73 μM.

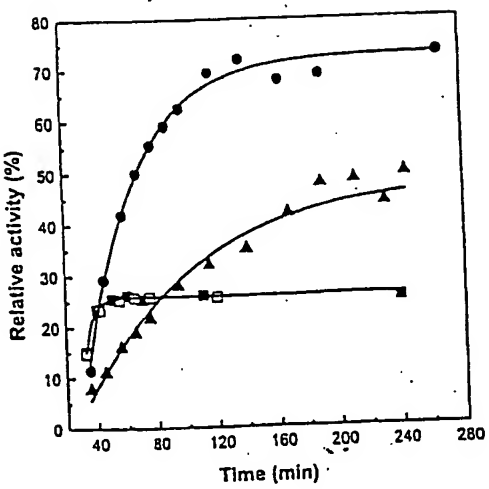


Fig. 5. Refolding kinetics of short-term denatured GAPDH in the presence of different concentrations of trigger factor. 2.73 μM GAPDH was denatured in 3 M GdnHCl for 10 s at 4 °C and reactivation started by rapid 50-fold dilution into 0.1 M K-Pi buffer containing different concentrations of trigger factor or cyclophilin. (■), (●), and (▲) were in the presence of 0, 10, and 45 μM trigger factor, respectively. (□) was in the presence of 15 μM cyclophilin. There was no detectable activity during the first 30 min of incubation at 4 °C.

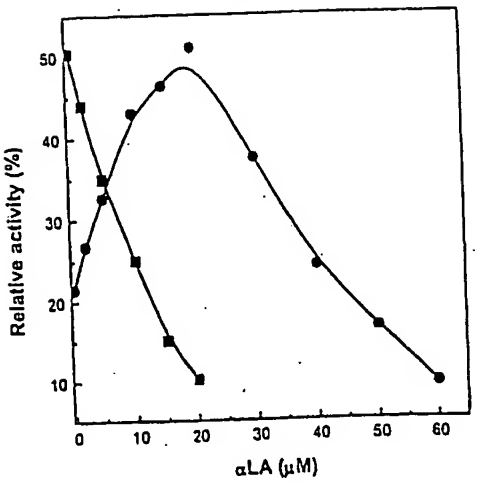


Fig. 6. Effect of reduced-αLA on trigger factor assisted reactivation of GAPDH. Refolding was initiated by 50-fold dilution of GdnHCl-denatured GAPDH into buffer containing different concentrations of reduced-αLA in the presence of 5.46 μM (■) or 45 μM (●) trigger factor, respectively. The GAPDH concentration was 2.73 μM.

reactivation of GAPDH increased to $(1.93 \pm 0.22) \times 10^{-1}$ min^{-1}, compared to $(7.68 \pm 0.72) \times 10^{-2}$ min^{-1} for longer denaturation times. The rates of reactivation of short-term denatured GAPDH in the presence of trigger factor were slower than that of spontaneous reactivation, as was found for long-term denaturation. Rate constants of reactivation in the presence of 0, 10, and 45 μM trigger factor were $(1.93 \pm 0.22) \times 10^{-1}$, $(2.8 \pm 0.25) \times 10^{-2}$, and $(1.32 \pm 0.12) \times 10^{-2}$ min^{-1}, respectively. Fifteen micromolars of cyclophilin had no effect on the kinetics of GAPDH refolding, indicating that the effect of trigger factor on GAPDH folding is not simply a consequence of its prolyl isomerase activity.

Competitive inhibition of trigger factor by a partially unfolded protein

Trigger factor has been shown to bind protein substrates in a nonspecific fashion (Valent et al., 1995; Hesterkamp et al., 1996; Deuerling et al., 1999) and, as a consequence, other unfolded proteins should interfere with this binding and inhibit its folding activity. Scholz et al. (1997) have used the reduced and carboxymethylated bovine αLA (RCM-LA) as a competitor for trigger factor catalyzed folding of RCM-T1. RCM-LA competes efficiently with RCM-T1 for binding to trigger factor. Here, we simply unfolded αLA with 5 mM DTT (Ewbank & Creighton, 1993a, 1993b) and investigated the effects of the reduced αLA on trigger factor assisted refolding of GAPDH. Our results (Fig. 6) show that trigger factor assisted refolding of GAPDH is suppressed when reduced αLA is added to the refolding buffer, at low ratios of trigger factor/GAPDH. The reactivation yields of GAPDH (2.73 μM) in the presence of trigger factor (5.46 μM) decreased with increasing concentration of reduced αLA and approached the level of spontaneous refolding when the concentration of reduced αLA was higher than 20 μM, which indicates that the reduced αLA competitively inhibits the folding activity of the trigger factor and also suggests GAPDH intermediates and the nonnative inhibitor bind in the same fashion and compete for the same polypeptide binding site on trigger factor. Interesting results were observed when the molar ratio of trigger factor to GAPDH was 16.5. The extent of reactivation increased with increasing concentration of reduced αLA and reached double the activity in the absence of αLA when the concentration of reduced αLA was 20 μM. Reactivation yields then decreased gradually with further increase in the concentration of reduced αLA and finally fell below the level of that in the absence of αLA. As shown in Figure 1, the reactivation of GAPDH was suppressed when molar ratios of trigger factor to GAPDH were >5. This is not caused by an increase in protein aggregation (Fig. 3A,B). At concentrations of trigger factor where refolding of GAPDH was inhibited, the binding of reduced αLA to trigger factor reduced the concentration of free trigger factor, and hence relieved the suppression of refolding of GAPDH. Further increase in the concentration of αLA finally reduced the amount of trigger factor available to bind GAPDH intermediates to the point where regain in activity was mainly from the pathway of spontaneous folding. Considering also that the refolding rate of GAPDH decreased continuously with increasing concentrations of trigger factor, this suggests that trigger factor forms complexes with GAPDH intermediates during folding, so that at high molar ratios of trigger factor to GAPDH folding is in effect arrested.

Inactive bound GAPDH can be partially rescued from trigger factor by a variety of treatments

To test the supposition that complexes were formed between trigger factor and GAPDH intermediates during folding, we added reduced αLA to the refolding mixture after renaturation, in an attempt to dissociate the complexes. An equal volume of reduced αLA was added to the refolding mixture of GAPDH in the presence of various concentrations of trigger factor to give a final αLA

concentration of 20 μM. As shown in Figure 7, there have been no obvious effects of αLA on the reactivation yields when the ratios of trigger factor to GAPDH were <5. However, when the ratios of trigger factor/GAPDH were >5, and the extent of reactivation began to fall with increasing ratios of trigger factor/GAPDH, addition of reduced αLA allowed a higher degree of reactivation, and the effect became more pronounced at higher ratios of trigger factor to GAPDH. A further 10% of activity was recovered after addition of reduced αLA when the trigger factor/GAPDH molar ratio was 16.5. The effect of different concentrations of reduced αLA on the additional recovery of activity of an equilibrated refolding mixture at a trigger factor/GAPDH ratio of 16.5 was also investigated. As shown in Figure 8, additional reactivity increased with increasing concentrations of reduced αLA and reached a constant value at about 20 μM αLA. The results support our suggestion that suppression of refolding of GAPDH at high concentrations of trigger factor is due to formation of complexes between trigger factor and GAPDH intermediates. Similar to that of trigger factor at high concentration, the formation of the complex of GroEL with the GAPDH folding intermediate suppresses both the reactivation and aggregation of GAPDH during folding was reported by Li et al. (1998). In the Discussion, a model for trigger factor assisted refolding of GAPDH is proposed to address why the amount of complexes formed is dependent on the amount of trigger factor present. In addition, we conducted a further two sets of experiments to investigate the properties of such complexes. First, the equilibrated refolding mixture of GAPDH in the presence of different concentrations of trigger factor was further diluted 10-fold with 0.1 M K-Pi buffer and incubated for a further 1.5 h at 25 °C before the activity was measured. As was found with addition of reduced αLA, further dilution had no effect on the reactivation yields when the trigger factor/GAPDH ratios were <5. When the ratios were >5, however, the extent of reactivation was further increased. An additional 8% of activity was recovered after this further dilution when the trigger factor/GAPDH molar ratio was 16.5. This suggests that complexes of GAPDH intermediates and trigger factor are in equilibrium between dissociated and associated forms, and that active GAPDH is obtained only after release from trigger factor. A decrease in the effective concentration of trigger factor by dilution would result in a readjustment of the equilibrium between bound and dissociated forms, allowing a further proportion of GAPDH intermediates to refold. When the equilibrated refolding mixture was diluted twofold with 0.1 M K-Pi buffer containing ethylene glycol (EG) to give a final EG concentration of 10% (v/v), there was likewise no obvious effect on the reactivation yields when the trigger factor/GAPDH ratios were <5. However, when the trigger factor/GAPDH ratios were >5, the extent of reactivation was significantly improved by EG, the effects increasing with increasing ratios of trigger factor/GAPDH. At a trigger factor/GAPDH molar ratio of 16.5, this further dilution of refolding mixture into a solution of 10% EG allowed an additional 22% recovery of activity (Fig. 7). The effect of different EG concentrations on the additional recovery of activity of an equilibrated refolding mixture with a trigger factor/GAPDH ratio of 16.5 is shown in Figure 9. Additional reactivity increased with increasing concentrations of EG and reached a constant value at about 10% EG. The effect of EG is mostly likely explained by its ability to disrupt hydrophobic interactions (Li & Zhou, 1997; Song et al., 1997). In general, molecular chaperones bind protein substrates through nonspecific hydrophobic interactions. If the complexes are maintained by this kind of force, bound GAPDH intermediates would dissociate from trigger factor when such forces are disrupted.

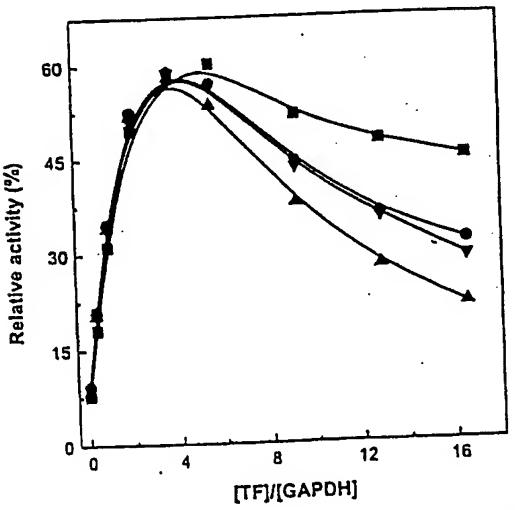


Fig. 7. Inactive bound GAPDH could be partially rescued from trigger factor by the competitor—reduced αLA. Denaturation of GAPDH was carried out in 3 M GdnHCl overnight at 25 °C. Refolding was carried out as described in Figure 1 before samples were taken for measurement of activity (▲). After this initial refolding period, the reactivation mixture was diluted twofold into 0.1 M K-Pi buffer containing 40 μM αLA (●), diluted 10-fold into 0.1 M K-Pi buffer only (▼), or diluted two fold into 0.1 M K-Pi buffer containing 20% (v/v) EG (■). The activity was measured after an additional 1.5 h at 25 °C. The concentration of GAPDH was 2.73 μM, and the ratios of trigger factor to GAPDH were as indicated.

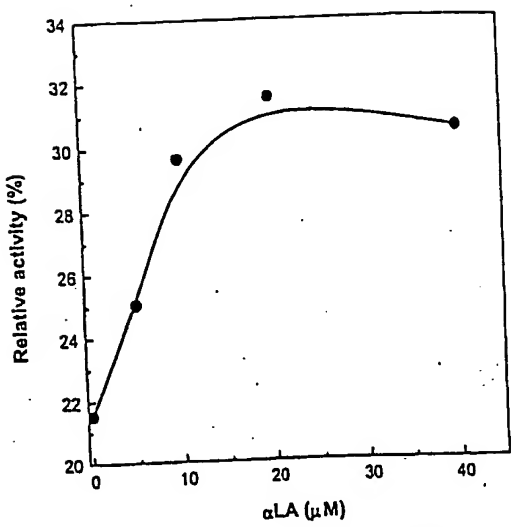


Fig. 8. Dependence of dissociation of trigger factor/GAPDH complex on reduced αLA concentration. Refolding was carried out as described in Figure 1, after which the refolding mixture was further diluted twofold into 0.1 M K-Pi buffer containing different amounts of reduced αLA to give final concentrations as indicated. GAPDH and trigger factor were 2.73 and 45 μM, respectively.

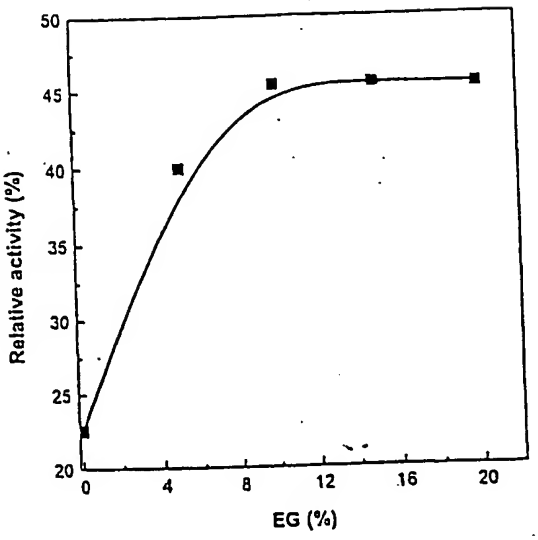


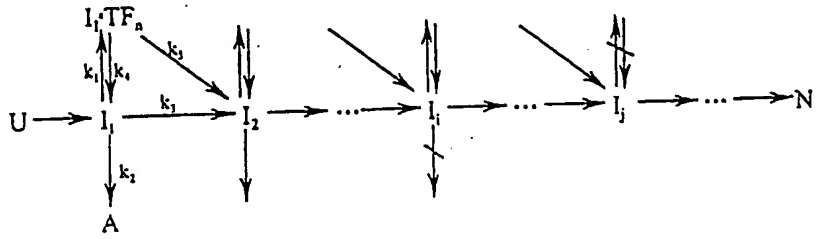
Fig. 9. Effect of EG concentration on the further recovery of activity of trigger factor-bound GAPDH. Refolding was carried out as described in Figure 1, after which the refolding mixture was further diluted twofold into the same buffer containing different concentrations of EG (the final concentrations of EG are indicated). GAPDH and trigger factor concentrations were 2.73 and 45 μM, respectively.

Discussion

Upon sudden dilution of GdnHCl-denatured GAPDH, the unfolded enzyme can either fold and assemble correctly to form the active tetrameric structure, or it may self-assemble incorrectly to form aggregates. Trigger factor hinders the incorrect association of the aggregation-prone species and thus favors the pathway to form active enzyme, improving reactivation but without being a part of the final functional structure. At the same time, the rates of assisted refolding of GAPDH are reduced by trigger factor with respect to the rate of spontaneous refolding after either long- or short-term denaturation. A comparison with cyclophilin as a reference foldase suggests that the high affinity toward unfolded protein chains is a requisite for the high efficiency of trigger factor in assisting protein folding. In its efficient binding to unfolded proteins, trigger factor resembles a chaperone. The strong binding of protein substrates appears also to decelerate their dissociation from trigger factor. Kern et al. (1995) have reported that the folding of human carbonic anhydrase II after a short 10 s unfolding pulse in 5 M GdnHCl is decelerated by cyclophilin because cyclophilin reverses the native proline configuration, but in the case of folding after long-term denaturation, when all unfolded molecules contained nonnative prolyl isomers, the reactivation is accelerated with increasing cyclophilin concentration. In the case of assisted refolding of GAPDH by trigger factor, the same possibility of prolyl isomerase activity of trigger factor exists. However, the deceleration is also found for longer denaturation times, supporting the suggestion that the deceleration of GAPDH refolding by trigger factor results not from prolyl isomerase activity, but from slow dissociation of GAPDH intermediates from trigger factor. In addition, trigger factor is effective in assisted GAPDH refolding only at stoichiometric concentrations. A catalytic quantity of trigger factor, which has been found to be effective in catalyzing prolyl *cis–trans* isomerization limited protein folding (Scholz et al., 1997), had no effect either on the reactivation yield or on preventing aggregation of GAPDH (data not shown). While we cannot rule out the possibility of an involvement of prolyl isomerase activity of trigger factor in improving the reactivation yields of GAPDH, the absence of any effect by cyclophilin, or catalytic quantities of trigger factor, indicates that in this case prolyl isomerase activity alone is not sufficient to assist folding. It could be due to bad accessibility of PPIases to the prolines in the unfolded molecules (Lang et al., 1987; Lin et al., 1988; Schmid et al., 1993). Instead, an additional, chaperone-like activity of trigger factor is required to be effective in improving reactivation yields.

In trigger factor assisted refolding, the degree of reactivation was found to increase as the concentration of trigger factor increased. However, this effect reached a maximum, after which increasing amounts of trigger factor resulted in lower reactivation yields. This increase then decreases in yield, and the correlated continuous increase in the $t_{1/2}$ of reactivation appears to be due to the strong binding of trigger factor to GAPDH intermediates. The nature of this interaction is most likely hydrophobic since it was greatly diminished by the presence of EG. EG increases the hydrophobicity of the solution, thereby decreasing the hydrophobic interaction between folding intermediate and trigger factor (Li & Zhou, 1997; Song et al., 1997). Rapid dilution of the GdnHCl denatured GAPDH to nondenaturing conditions allowed at least three noncovalent processes to occur: intramolecular organization of peptide segments (folding), intermolecular assembly of hydrophobic surfaces (aggregation), and formation of the trigger factor/GAPDH complex. The result of this competition may be determined by either kinetic or thermodynamic factors. The general pathway of trigger factor assisted folding can be expressed by Scheme 1.

Scheme 1 presents a hypothetical mechanism for the events that could occur upon dilution of GdnHCl denatured GAPDH in the presence of trigger factor. The central left-to-right path illustrates



Scheme 1.

the route of spontaneous folding, initiated from the unfolded state U. GAPDH refolding involves the formation of an intermediate I_1, which is not enzymatically active and can partition into an inactive aggregate A; complex with trigger factor $I_1 \cdot TF_n$; or form active product N. n is the number of trigger factor molecules binding to a single substrate molecule in the case that there are two or more hydrophobic sites on unfolded GAPDH that can be anchored to trigger factor molecules. I_1, I_2, to I_{i-1} represent a series of intermediates during refolding that have the potential ability to take all three pathways. In this scheme, trigger factor assisted folding requires repeated binding-and-release cycles, although not all intermediates are necessarily involved in the cycle. The higher the concentration of trigger factor, the larger the number of binding-release cycles. I_i to the last intermediate before the native state represents late intermediates with most hydrophobic surfaces buried within the molecule and so is no longer prone to aggregation. The intermediates after I_j, due to their compact structure, would no longer be substrates for trigger factor. In the proposed mechanism, some folding of GAPDH is likely to occur during association with trigger factor, but the final active enzyme can only be formed in the unbound state. It should be noted that assembly of GAPDH subunits and possible *cis–trans* isomerization of prolyl peptide bonds are not taken into consideration.

Materials and methods

Purification and activity determinations of rabbit muscle GAPDH were as described previously (Liang et al., 1990). An absorbance coefficient of ϵ_{280nm} = 144,000 M^{-1} cm^{-1} was used for holoenzyme concentration determination. For trigger factor, a value for ϵ_{280nm} of 15,930 M^{-1} cm^{-1} was calculated using the procedure of Gill and von Hippel (1989), and it was purified as described (Stoller et al., 1995). Cyclophilin was prepared from porcine kidney according to Kofron et al. (1991). The specific constant of final product is about 1.9×10^7 M^{-1} s^{-1}.

PPIase activity was assayed using the chymotrypsin-coupled method (Fischer et al., 1984). Tetrapeptide (succinyl-Ala-Ala-Pro-Phe-4-nitroanilide) was purchased from Peptide Institute Inc. (Osaka, Japan). Chymotrypsin (type VII), α-lactalbumin (αLA) (type III), and β-NAD were obtained from Sigma (St. Louis, Missouri). DL-Glyceraldehyde-3-phosphate (DL-GAP) was prepared from the water-insoluble barium salt of the diethylacetal (Sigma) by treatment as described (Scheek & Slater, 1982). DL-Dithiothreitol (DTT) was a Gibco product, while GdnHCl was purchased from ICN Biomedicals (Cosa Mesa, California). All other chemicals were local products of analytical grade. In all experiments, 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 5 mM DTT was employed.

Denaturation of GAPDH was carried out by incubation of the enzyme in 3 M GdnHCl with 5 mM DTT either overnight at 4 °C or for only 10 s at 4 °C. Reactivation of denatured GAPDH was carried out by dilution of the denatured enzyme into phosphate buffer containing different concentrations of trigger factor or cyclophilin. The reactivation mixture was kept at 4 °C for 30 min and then for a further 3 h at 25 °C to allow reactivation go to completion before the final yield of GAPDH activity was determined, which was performed at 25 °C using a Beckman DU7500 instrument. The time course of reactivation of GAPDH was followed by determining activities of samples withdrawn at the indicated times. The kinetic data were analyzed by fitted to a single-exponential function. GAPDH itself is stable when subjected to the same treatment without denaturant. Aggregation of GAPDH upon dilution was monitored continuously at 20 °C by 90° light scattering at 488 nm in a Hitachi F-4500 spectrofluorometer. All measurements were repeated several times, and the rate constants obtained were well reproducible.

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References

Cai H, Wang CC, Tsou CL. 1994. Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. *J Biol Chem 269*:24550–24552.

Crooke E, Guthrie B, Lecker S, Lill R, Wickner W. 1988. ProOmpA is stabilized for membrane translocation by either purified *E. coli* trigger factor or canine signal recognition particle. *Cell 54*:1003–1011.

Crooke E, Wickner W. 1987. Trigger factor: A soluble protein that folds pro-OmpA into a membrane-assembly-competent form. *Proc Natl Acad Sci USA 84*:5216–5220.

Deuerling E, Schulze-Specking A, Tomoyasu T, Mogk A, Bukau B. 1999. Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature 400*:693–696.

Ewbank JJ, Creighton TE. 1993a. Pathway of disulfide-coupled unfolding and refolding of bovine α-lactalbumin. *Biochemistry 32*:3677–3693.

Ewbank JJ, Creighton TE. 1993b. Structural characterization of the disulfide folding intermediates of bovine α-lactalbumin. *Biochemistry 32*:3694–3707.

Fischer G, Bang H, Mech C. 1984. Nachweis einer Enzymkatalyse für die cis–trans-Isomerisierung der Peptidbindung in prolinhaltigen Peptiden. *Biomed Biochim Acta 43*:1101–1111.

Freskgård PO, Bergenhem N, Jonsson BH, Svensson M, Carlsson U. 1992. Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. *Science 258*:466–468.

Gill SC, von Hippel PH. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem 182*:319–326.

Göthel SF, Scholz C, Schmid FX, Marahiel MA. 1998. Cyclophilin and trigger factor from *Bacillus subtilis* catalyze in vitro protein folding and are necessary for viability under starvation conditions. *Biochemistry 37*:13392–13399.

Guthrie B, Wickner W. 1990. Trigger factor depletion or overproduction causes defective cell division but does not block protein export. *J Bacteriol 172*:5555–5562.

Hesterkamp T, Hauser S, Lütcke H, Bukau B. 1996. *Escherichia coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. *Proc Natl Acad Sci USA 93*:4437–4441.

Kandror O, Sherman M, Moerschell R, Goldberg AL. 1997. Trigger factor associates with GroEL in vivo and promotes its binding to certain polypeptides. *J Biol Chem* 272:1730–1734.

Kandror O, Sherman M, Rhode M, Goldberg AL. 1995. Trigger factor is involved in GroEL-dependent protein degradation in *Escherichia coli* and promotes binding of GroEL to unfolded proteins. *EMBO J 14*:6021–6027.

Kern G, Kern D, Schmid FX, Fischer G. 1995. A kinetic analysis of the folding of human carbonic anhydrase II and its catalysis by cyclophilin. *J Biol Chem 270*:740–745.

Kofron JL, Kuzmič P, Kishore V, Colón-Bonilla E, Rich DH. 1991. Determination of kinetic constants for peptidyl prolyl *cis–trans* isomerases by an improved spectrophotometric assay. *Biochemistry 30*:6127–6134.

Lang K, Schmid FX, Fischer G. 1987. Catalysis of protein folding by prolyl isomerase. *Nature 329*:268–270.

Li XF, Zhou JM. 1997. Unfolding and aggregation-associated changes in the secondary structure of D-glyceraldehyde-3-phosphate dehydrogenase during denaturation by guanidine hydrochloride as monitored by FTIR. *Biospectroscopy 3*:121–129.

Li XL, Lei XD, Cai H, Li J, Yang SL, Wang CC, Tsou CL. 1998. Binding of a burst-phase intermediate formed in the folding of denatured D-glyceraldehyde-3-phosphate dehydrogenase by chaperonin 60 and 8-anilino-1-naphthalene-sulphonic acid. *Biochem J 331*:505–511.

Liang SJ, Lin YZ, Zhou JM, Tsou CL, Wu PQ, Zhou ZK. 1990. Dissociation and

aggregation of D-glyceraldehyde-3-phosphate dehydrogenase during denaturation by guanidine hydrochloride. *Biochim Biophys Acta 1038*:240–246.

Lin LN, Hasumi H, Brandts JF. 1988. Catalysis of proline isomerization during protein-folding reactions. *Biochim Biophys Acta 956*:256–266.

Lyon WR, Gibson CM, Caparon MG. 1998. A role for trigger factor and an rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*. *EMBO J 17*:6263–6275.

Scheek RM, Slater EC. 1982. Glyceraldehyde-3-phosphate dehydrogenase from *rabbit* muscle. *Methods Enzymol 89*:305–309.

Schmid FX, Mayr LM, Mücke M, Schönbrunner ER. 1993. Prolyl isomerases: Role in protein folding. *Adv Protein Chem 44*:25–66.

Scholz C, Stoller G, Zarnt T, Fischer G, Schmid FX. 1997. Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding. *EMBO J 16*:54–58.

Song JL, Quan H, Wang CC. 1997. Dependence of the anti-chaperone activity of protein disulphide isomerase on its chaperone activity. *Biochem J 328*:841–846.

Stoller G, Rücknagel KP, Nierhaus KH, Schmid FX, Fischer G, Rahfeld JU. 1995. A ribosome-associated peptidyl-prolyl *cis/trans* isomerase identified as the trigger factor. *EMBO J 14*:4939–4948.

Teipel JW, Koshland DE. 1971. Kinetic aspects of conformational changes in proteins. I. Rate of regain of enzyme activity from denatured proteins. *Biochemistry 10*:792–798.

Valent QA, Kendall DA, High S, Kusters R, Oudega B, Luirink J. 1995. Early events in preprotein recognition in *E. coli*: Interaction of SRP and trigger factor with nascent polypeptides. *EMBO J 14*:5494–5505.